LOCATION OF CHROMOPHORIC RESIDUES IN RIBONUCLEASE T_1 BY SOLVENT PERTURBATION DIFFERENCE SPECTROSCOPY

M. K. Campbell, S. Shipp and E. Jantzen

Carr Laboratory, Department of Chemistry

Mount Holyoke College, South Hadley, Massachusetts

Received August 11,1976

SUMMARY

The degree to which aromatic amino acid residues in ribonuclease T_1 are accessible to solvent has been determined by the technique of solvent perturbation ultraviolet difference spectroscopy. Two of the nine tyrosine residues are at the surface and freely accessible to solvent. The single tryptophan residue and two to three additional tyrosine residues are close enough to the surface to be available for long range interactions with solvent. Both groups of aromatic residues are available to play a role in recognition and binding of substrate.

The enzyme RNase T_1 [1] (EC 3.1.4.8) from Aspergillus oryzae is particularly well suited for use in studies of recognition of nucleic acids by specific proteins. It is a small protein, reasonably well characterized, of known primary structure, and highly specific for guanosine residues as the site of degradation of RNA (1). This enzyme has subsites for attachment of oligomeric substrates, and these subsites may involve the aromatic amino acid residues (2). No detailed crystallographic study of RNase T_1 has appeared, but circular dichroism studies indicate that the percentage of both α-helical and β-pleated sheet type structure is low (3,4). The question, then is whether a significant portion of the aromatic residues, including one tryptophan and nine tyrosines (1) are close enough to the surface of the enzyme to interact with the RNA bases. It is not enough to answer this question for the crystalline enzyme; a solution study is needed as well, and we have chosen the method of solvent perturbation difference spectroscopy for this purpose.

In this method, non-denaturing solvents are used, and the overall conforma-

1. Abbreviations: RNase, ribonuclease: DMSO, dimethyl sulfoxide.

tion of the protein is not changed (5). The use of long and short range perturbants allows us to distinguish between the chromophores directly on the surface and those just below it (6). Aromatic rings in such locations would be available for direct or longer range interactions with RNA bases.

MATERIALS AND METHODS

Crystalline RNase T_1, prepared by Sankyo Ltd., was purchased from Calbiochem. Preliminary chromatographic testing had shown it to be homogeneous, and it was used without further purification. N-acetyl-L-tyrosine ethyl ester, from Sigma, and N-acetyl-L-tryptophan ethyl ester, from Cyclo, were used as spectroscopic standards. All perturbants and buffer salts were analyzed reagent grade.

The method of solvent perturbation difference spectroscopy in the wavelength range 250-310 nm was essentially that described by Herskovits and Laskowski (5), using a Cary 17 Spectrophotometer. All spectra were obtained with the instrument operating under conditions described as optimal by Herskovits and Sorensen (7). All runs were done in triplicate, except for sucrose and glucose, which were duplicates.

In all experiments the samples contained enough KCl to give a final ionic strength of 0.2. The samples run at pH 5.10 contained 100 mM sodium acetate; the ones run at pH 6.96 contained 100 nM potassium phosphate. The final concentration of all perturbants except deuterium oxide, glucose and sucrose was 20% v/v. For glucose and sucrose, the final concentration was 20% w/v, and for D_2O, 90% v/v. The pH values chosen were those of maximum enzyme activity (pH 7) and optimal binding of guanosine monophosphate inhibitors to the enzyme (pH 5) (1).

RESULTS AND DISCUSSION

The standard for "complete exposure" to solvent of the tyrosine and tryptophan chromophores was a mixture of the two model compounds, N-acetyl-L-tyrosine ethyl ester and N-acetyl-L-tryptophan ethyl ester in a 9:1 molar ratio, reflecting the ratio of tyrosine to tryptophan residues in RNase T_1. The concentration of the model compound mixture in each perturbant was chosen to give the same concentration of chromophores on a residue basis as that of the enzyme itself in the same perturbant. The overall degree of exposure of chromophores is then the percentage of the change in absorbance of the standard exhibited by the enzyme at a given wavelength (5). A second standard involved the use of simultaneous equations due to Herskovits (8) to calculate molar difference extinction coefficients for the two separate amino acid esters.

$$\Delta\varepsilon_{292-294}(\text{protein}) = a\,\Delta\varepsilon_{292-294}(\text{Trp}) + b\,\Delta\varepsilon_{292-294}(\text{Tyr}) \quad (1)$$

$$\Delta\varepsilon_{286-288}(\text{protein}) = a\,\Delta\varepsilon_{286-288}(\text{Trp}) + b\,\Delta\varepsilon_{286-288}(\text{Tyr}) \quad (2)$$

In these equations, the $\Delta\varepsilon$ are the molar difference extinction coefficients, at the wavelength indicated by the subscript, for the model compounds or for the protein. The coefficients a and b are the numbers of tryptophan and tyrosine chromophores, respectively, which are exposed to solvent. Both methods of calculation of the degree of exposure of chromophores were used at pH 7, and the first method only was used in the less extensive experiments at pH 5 to see whether the general trend of results remained the same. When both methods of calculation were used, the results were in agreement to within experimental error.

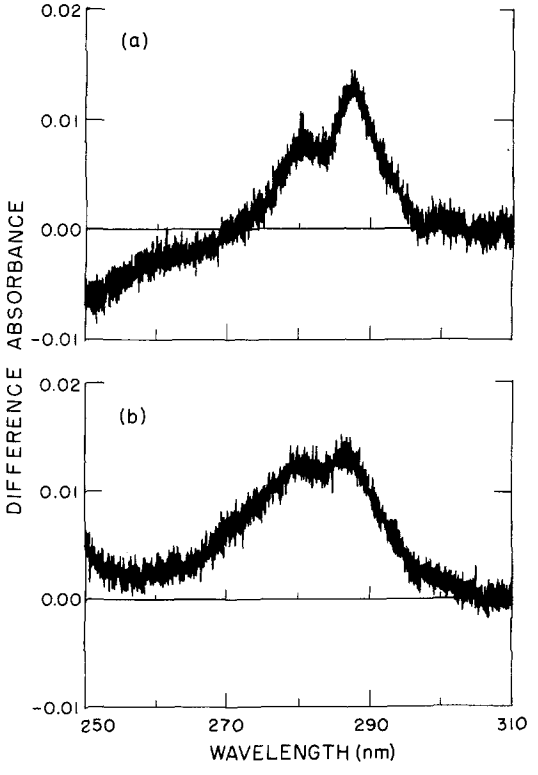

Figure 1: Perturbation difference spectra of ribonuclease T_1 (a) in 20% v/v DMSO, protein concentration 0.45 mg/ml (b) in 20% v/v glycerol protein concentration 0.47 mg/ml. The solvent was 100 nM potassium phosphate (pH 6.96) adjusted to 0.2M ionic strength with KCl. The temperature was 23 ± 0.1°C.

Figure 1 shows typical examples of the spectral results obtained with the enzyme, and are direct tracings from the chart paper. The experimentally observed difference absorbance is presented here, rather than the calculated molar difference extinction coefficient for DMSO and for glycerol, typical short range and long range perturbants respectively. Table 1 presents a summary of the results with all perturbants at both pH values.

The first general observation is that there was a greater degree of perturbation of tyrosine residues at pH 7 by long range perturbants than by short range ones. Of the short range perturbants, the two larger ones affected only two of the nine tyrosines, which can be considered to be at the surface of the molecule. Methanol, the third and smallest of the short range perturbants, affected three tyrosine residues, indicating that a third tyrosine residue could be located in a crevice or cleft, over and above the two on the surface. In addition to these three tyrosine residues, there were several affected by long range perturbants, the number of which did not vary in any markedly regular way with the size of the perturbant. D_2O is considered to be the most penetrating of the long range perturbants, and ethylene glycol the least (6). Our results reflected this property with a total of 5.60 and 3.60 tyrosine residues affected respectively. The results for the other three long range perturbants lay within a narrow range, 3.90 to 4.50 tyrosines overall. We interpret these data to indicate that on the average another one or two tyrosine residues are available for long range interactions, such as those between π systems of their phenol rings and those of the RNA bases.

The tryptophan exposure at pH 7 did not vary greatly with the size or range of the perturbants used. The average value for all eight perturbants was 0.42 groups exposed, compared to the average of 0.35 groups with the three short range perturbants, and 0.46 groups with the five long range perturbants. The single tryptophan residue of RNase T_1 can thus be considered partially exposed and able to contribute to recognition or binding.

Similar experiments done at pH5 with all the short range and three of the

TABLE 1 SUMMARY OF CHROMOPHORE EXPOSURE FOR RNase T_1

PERTURBANT	EFFECTIVE DIAMETER (A) (a)	RANGE	NUMBER OF GROUPS PERTURBED AT pH 7		OVERALL DEGREE OF PERTURBATION	
			Tyr	Trp	pH 7	pH 5
Methanol	2.8	Short	2.90	0.23	0.31	0.32
DMSO	4.0	Short	1.90	0.55	0.22	0.26
Polyethylene Glycol	9.2	Short	2.10	0.28	0.15	0.26
D_2O	2.0	Long	5.60	0.55	0.64	(b)
Ethylene Glycol	4.4	Long	3.60	0.48	0.40	0.37
Glycerol	5.2	Long	4.50	0.59	0.44	0.37
Glucose	7.2	Long	4.10	0.22	0.41	(b)
Sucrose	9.4	Long	3.92	0.44	0.44	0.53

(a) Taken from Reference 6
(b) Not done at pH 5

five long range perturbants used at pH 7 indicated that the trend in the overall degree of perturbation remained the same, and it is the overall trend that is of importance here. These experiments show that there is one group of tyrosines at the surface of the molecule, and another group readily accessible to the surface. The tryptophan is in an environment similar to that of the second group of tyrosines.

The guanosine moiety at the point of hydrolytic attack is bound more strongly to RNase T_1 than the other bases in a given segment of RNA (2). Invoking the "selective bookmark" hypothesis of Gabbay (9), it is possible to assign the two freely accessible tyrosine residues to the primary recognition site. The sequence of residues 56 to 59 in RNase T_1 is tyrosine - tyrosine - glutamic acid - tryptophan, and the glutamic acid is required for enzymatic activity (1). Pongs (10, 11) has suggested on the basis of fluorescence results that the two tyrosine residues which occur in this sequence are in a highly polar environment, and that the tryptophan is in a relatively non-polar one. Assuming that tyrosines 56 and 57 are the two at the surface of the molecule, the remaining accessible tyrosine residues, whether in a cleft or just below the surface, and the tryptophan can be assigned a role in the weaker binding sites involving other bases. Histidines 40 and 92 have also been implicated as a part of the active site (12), and one of these, number 40, is also flanked by tyrosines numbers 38 and 42, which could be part of the group near the surface. The precise identity of the tyrosine residues which are at or near the surface of the enzyme molecule is not, of course, established unambiguously by this work. What has been established is that there are enough aromatic residues at the right level of accessibility and in two subgroups, like the binding sites themselves to play a role in the recognition of substrate by the enzyme. Work is continuing in this laboratory to elucidate further the mode of interaction between nucleic acid bases and specific enzymes.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Institutes of Health and Research Corporation. The purchase of the Cary spectrophotometer was supported in part by a Research Equipment Grant from the National Science Foundation. SS was a National Science Foundation Undergraduate Research participant.

REFERENCES

1. Takahashi, K., Uchida, T., and Egami, F. (1970) Advan. Biophys., 1, 53-98.
2. Walz, F.G., and Terenna, B. (1976) Biochemistry, 15, 2837-2842.
3. Sander, C., and Ts'o, P.O.P. (1971) Biochemistry, 10, 1953-1965.
4. Yamamoto, Y., and Tanaka, J. (1970) Biochem. Biophys. Acta, 207, 522-531.
5. Herskovits, T.T., and Laskowski, M. (1962) J. Biol. Chem., 237, 2481-2492.
6. Kronman, M.J., and Robbins, F.M. (1970) in Fine Structure of Proteins and Nucleic Acids, Fasman, G.D., ed., pp. 282-287, Marcel Deckker, New York.
7. Herskovits, T.T., and Sorensen, M. (1968) Biochemistry, 7, 2523-2542.
8. Herskovits, T.T. (1967) Methods Enzymol., 11, 748-749.
9. Gabbay, E.J., Sanford, K., Baxter, C.S. and Kapicak, L. (1973) Biochemistry 12, 4021-4028.
10. Pongs, O. (1970) Biochemistry, 9, 2316-2321.
11. Pongs, O. (1970) Biochem. Biophys. Res. Commun., 38, 431-437.
12. Takahashi, K. (1973) J. Biochem. (Tokyo), 74, 1279-1288.